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HMGB1 as a cytokine and therapeutic target

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HMGB1 is an abundant nuclear and cytoplasmic protein present in mammalian cells. It is traditionally known as a DNA binding protein involved in maintenance of nucleosome structure and regulation of gene transcription. Beyond these intracellular roles, we recently discovered that HMGB1 is released from activated macrophages and functions as a late mediator of lethal endotoxemia. Addition of HMGB1 to macrophage cultures activates cytokine release. When released into the extracellular milieu, HMGB1 causes systemic inflammatory responses including acute lung injury, epithelial barrier dysfunction, and death. Passive immunization with anti-HMGB1 antibodies confers significant protection against lethality induced by LPS administration and sepsis caused by cecal perforation in mice. Truncation of HMGB1 into individual structural domains revealed that the HMGB1 A box, a DNA-binding motif, specifically antagonizes the activity of HMGB1 and rescues mice from lethal sepsis caused by cecal perforation. Thus, strategies that target HMGB1 with specific antibodies or antagonists have potential for treating lethal systemic inflammatory diseases characterized by excessive HMGB1 release.

HMGB1 AS A CELL-ASSOCIATED PROTEIN

High mobility group box-1 (HMGB1, previously HMG-1) was first described by Goodwin *et al.* as a non-histone nuclear protein with high electrophoretic mobility.[2] Structurally, HMGB1 is composed of three domains: two homologous DNA-binding motifs termed A and B boxes, each made up of approximately 80 amino acids, and a negatively charged C-terminus.[3–5] Intracellular roles of HMGB1 include stabilizing nucleosome structure, and facilitating DNA bending.[3,4] HMGB1 is a surface membrane protein in some cells, where it can mediate neurite outgrowth, smooth muscle cell chemotaxis, and tumor cell metastasis.[6–8]

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HMGB1 AS A CYTOKINE

Discovery of HMGB1 as a cytokine

In an effort to broaden the therapeutic window for treatment of sepsis and endotoxemia, we searched for macrophage-derived factors that are released 'late' during endotoxemia. HMGB1 is released from macrophage-like RAW 264.7 cells 16 h after LPS exposure, but not at earlier time points (Fig. 1).[9] Serum HMGB1 levels increase in mice 16–32 h after LPS stimulation, and passive immunization of anti-HMGB1 antibodies confers significant protection against lethality, indicating that HMGB1 is a delayed mediator of endotoxemia.

In vitro *studies*

HMGB1 is a secreted product from macrophages, monocytes and pituicytes activated by exposure to LPS, TNF or IL-1β.[9,10] It is also passively released from necrotic or damaged cells.[6,7,11] Cells deficient in HMGB1 by gene knockout induce significantly less TNF release from bone marrow cells as compared to wild-type, indicating that cell-associated HMGB1 is a critical stimulus to inflammation at sites of cell death.[11] Recent studies using

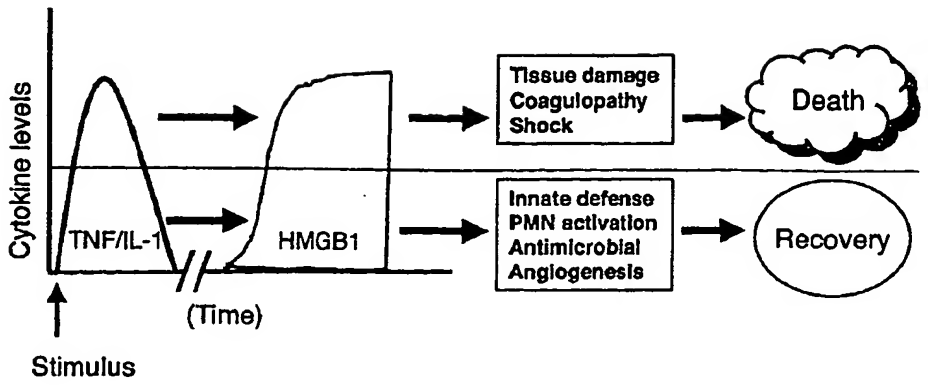

Fig. 1. Early and late cytokine mediators in inflammation. Stimuli from bacterial components (*i.e.* LPS, enterotoxins, TSST-1) activate macrophages to sequentially release early (*e.g.* TNF, IL-1) and late (*e.g.* HMGB1) cytokines. A small amount of these cytokines is beneficial to the host as this enhances innate defenses against pathogens, including activation of neutrophils, antimicrobial activity and stimulation of angiogenesis that lead to recovery from the invasion. A large amount of these cytokines causes tissue damage and even death.

immunofluorescent analysis indicated that HMGB1 is relocated from the nucleus to cytoplasmic organelles in LPS-activated monocytes, and subsequently secreted via a non-classical, vesicle-mediated secretory pathway.[12] Like other pro-inflammatory cytokines, HMGB1 is a potent activator of cytokine release from cultured human monocytes. Addition of HMGB1 to monocyte cultures stimulates the release of TNF, IL-1β, IL-1α, IL-1Ra, IL-6, IL-8, MIP-1α, and MIP-1β.[13] Taken together, these studies indicate that HMGB1 is a potent pro-inflammatory cytokine (Table 1).

Animal studies

Administration of even low doses of HMGB1 (5–50 μg/mouse) is associated with fever, weight loss, pilo-erection and reduced food intake.[9,14] Injection of higher doses (50–500 μg/mouse) is lethal. HMGB1 is toxic to LPS-resistant C3H/HeJ mice, indicating that HMGB1 mediates lethality in the absence of LPS signaling.[9] Intratracheally administered HMGB1 causes acute lung injury as manifested by neutrophil accumulation, lung edema and increased pulmonary cytokine levels.[15]

Table 1. Cytokine activity of HMGB1

Cell	**HMGB1**	**Reference**
Macrophages/ monocytes	1. Increases TNF mRNA and protein release, increases IL-1α, IL-1β, IL-1RA, IL-6, IL-8, MIP-1α and MIP-1β release	Andersson *et al.*[13]
	2. Serum release after LPS stimulation	Wang *et al.*[9]
Neutrophils	Increases TNF, IL-1β and MIP-2 release	Abraham *et al.*[15]
Epithelial cells	Increases enterocyte permeability	Sappington *et al.*[16]
Smooth muscle cells	Causes chemotaxis	Degryse *et al.*[6]
Tissue/animal	**Physiological responses**	**Reference**
Brain	Induces fever and anorexia	Agnello *et al.*[14]
Intestine	Induces intestinal barrier dysfunction	Sappington *et al.*[16]
Lung	Causes increased pulmonary levels of TNF, IL-1β and MIP-2, lung edema and neutrophil accumulation	Abraham *et al.*[15]
Mice	1. Serum release after LPS stimulation	
	2. Causes death	Wang *et al.*[9]
Human	1. Serum release in patients with septic or hemorrhagic shock	Wang *et al.*[9] Ombrellino *et al.*[17]
	2. Elevated levels in synovial fluid in patients with rheumatoid arthritis	Kokkola *et al.*[18]

Treatment with anti-HMGB1 antibodies in mice exposed to intratracheal LPS significa[redacted]decreases lung edema and neutrophil accumulation.[redacted]-HMGB1 antibodies do not significantly suppress LPS-induced elevation of pulmonary cytokines, indicating that the protective effects of HMGB1 antibodies against LPS-induced lung injury are specific.[15] HMGB1 increases the permeability of cultured Caco-2 enterocytic cells and impairs intestinal barrier function in mice.[16] Collectively, these data suggest that HMGB1 mediates lethal toxicity, in part through acute lung injury and gut barrier dysfunction (Table 1).

Clinical findings

Serum levels of HMGB1 are elevated in patients with sepsis[9] and hemorrhagic shock,[17] and in the synovial fluid of patients with rheumatoid arthritis.[18] Serum HMGB1 levels in normal humans are less than 5 ng/ml, but increase significantly in critically ill septic patients (50–200 ng/ml); the levels in septic patients are higher in non-survivors than in survivors.[9] In a non-septic patient with hemorrhagic shock, serum HMGB1 levels increased within 24 h after the onset of hemorrhagic shock, remained elevated for 72 h, then decreased as the clinical condition improved by 96 h.[17] Synovial fluid obtained from patients with rheumatoid arthritis showed elevated HMGB1 levels (1–10 μg/ml) in 12 out of 14 samples.[18] Thus, HMGB1 may play a role in the pathogenesis of human disease including sepsis, hemorrhagic shock, and chronic arthritis.

THE PRO-INFLAMMATORY ACTIVITY OF HMGB1 MAPS TO THE B BOX

To elucidate the structure–function relationship of HMGB1, we created truncated HMGB1 proteins by the PCR method, subcloned the PCR products into the expression vector and expressed these mutant proteins in *Escherichia coli*. The recombinant proteins were purified and screened for cytokine-stimulating activity in cultured macrophages. Truncation of HMGB1 into individual structural domains revealed that a mutant containing the B box retains the TNF-stimulating activity of HMGB1, indicating that the pro-inflammatory domain of HMGB1 maps to the B box (Yang *et al.*, submitted). This observation is further supported by studies using chemically synthesized B box, which also stimulates TNF release. A box protein, which shares 30% structural homology with B box,[3–5] does not significantly stimulate TNF release. Affinity purified anti-B box antibodies significantly suppress TNF stimulation induced by B box, giving evidence that the TNF-stimulating effects of B box are specific.

In vivo, B box is highly lethal in a D-galactosamine sensitized mouse model;[19,20] B bo[redacted]iated dose-dependent toxicity within 7–8 h after [redacted]nistration in Balb/C mice. B box is also lethal to LPS-resistant C3H/HeJ mice, indicating that B box is toxic in the absence of LPS signaling. Moreover, passive immunization with anti-B box antibodies in endotoxin-sensitive mice significantly protects against LPS lethality, indicating that selective inhibition of B box attenuates the toxicity of endogenous HMGB1. Thus, B box alone is sufficient to recapitulate the cytokine-stimulating effects of full-length HMGB1. Further analysis of B box and the cellular receptor(s) with which it interacts will help to guide future development of HMGB1 inhibitors.

A BOX ANTAGONIZES HMGB1-INDUCED CYTOKINE ACTIVITY

In vitro, we found that A box dose-dependently inhibited HMGB1-mediated TNF release in macrophage cultures. A box displaced saturable [^{125}I]-HMGB1 cell surface binding to macrophages, indicating that A box competes for surface binding with HMGB1. To determine whether A box can neutralize the toxicity of HMGB1 *in vivo*, Balb/C mice were subjected to either LPS injection or cecal ligation and puncture (CLP[21]). A box significantly rescued mice from the lethality induced by LPS or cecal perforation; importantly, A box could be administered as late as 24 h after cecal perforation and still successfully rescued mice from lethal sepsis (manuscript submitted). Thus, A box acts as an antagonist of HMGB1 *in vitro* and *in vivo*, and may be used as a therapeutic to reverse the course of established lethal sepsis.

FUTURE DIRECTIONS

The discovery of HMGB1 as a potent, monocyte/macrophage-derived, late-acting cytokine mediator of endotoxemia and sepsis has initiated a new field of investigation for the development of therapeutics in the treatment of sepsis. This also raises several important questions regarding the mechanisms that regulate HMGB1 release from cells, the identity of cell surface receptors and the downstream signal transduction pathways. The pursuit of these questions will help understanding HMGB1 action, and may eventually lead to the development of anti-HMGB1 in therapeutics for the treatment of inflammation.

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